

Spring 2014

Effects of keratin filaments on ERK signaling during Fas-induced death of cervical cancer (HeLa) cells

Amanda Berger

University of New Hampshire - Main Campus, ajj94@wildcats.unh.edu

Follow this and additional works at: <https://scholars.unh.edu/honors>

 Part of the [Cancer Biology Commons](#)

Recommended Citation

Berger, Amanda, "Effects of keratin filaments on ERK signaling during Fas-induced death of cervical cancer (HeLa) cells" (2014).
Honors Theses and Capstones. 204.
<https://scholars.unh.edu/honors/204>

This Senior Honors Thesis is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Honors Theses and Capstones by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.

**Effects of keratin filaments on ERK signaling during
Fas-induced death of cervical cancer (HeLa) cells**

Honors Senior Thesis
May 2014

Undergraduate: Amanda Berger
Major: Biomedical Science, Medical and Veterinary Option

Faculty Advisor: Dr. David H. Townson
Department: Molecular, Cellular and Biomedical Sciences

Table of Contents

Abstract...	1
Introduction...	2
Materials and Methods...	9
Results...	11
Discussion...	12
Figures...	14
References...	17
Appendix I: Treatment Diagram for 6-well Culture Plates...	20
Appendix II: Cell Lysates Protocol...	21
Appendix III: Western Blotting Protocol...	22
Appendix IV: Immunodetection Protocol...	23
Appendix V: Treatment Diagram for 96-well Culture Plates...	24
Appendix VI: In-Cell Western Protocol...	25
Appendix VII: Undergraduate Research Conference Poster...	26

Abstract

Survival of cancer cells is influenced by a variety of factors, including physical elements such as keratin filaments. We know HeLa cells containing or lacking keratin 8/18 intermediate filaments (K⁺ and K⁻ cells, respectively) are more sensitive to the death-inducing effects of Fas agonist compared to the cytokines tumor necrosis factor alpha (TNF- α) or TNF-related apoptosis-inducing ligand. Additionally, K⁻ cells are more sensitive to Fas-induced death than K⁺ as shown by previous studies using mitochondrial activity and caspase activation assays. In the current study we tested the hypothesis that keratin filaments associate with the mitogen activated protein kinase (MAPK) cascade to protect cells from Fas-induced death. To do this, K⁺ and K⁻ cells were exposed to Fas agonist in the absence or presence of human epidermal growth factor (EGF) (known to stimulate MAPK) and then downstream phosphorylation of extracellular signal-regulated kinase (pERK) was measured. Fas agonist reduced pERK expression in both cell types (~32% less pERK compared to non-EGF-treated controls, n= 3 expts.). Conversely, EGF (50ng/ml) reversed this outcome, but again did so equally in K⁺ and K⁻ cells. Intriguingly, K⁻ cells were more responsive to EGF stimulation alone than K⁺ cells, regardless of EGF dose (pERK ~27% higher in K⁻ than K⁺ cells, n= 3 expts.). The results suggest keratin 8/18 filaments do associate with the MAPK cascade to influence cell survival. Supported by the Hamel Center for Undergraduate Research (AB) and the COLSA Karabelas Fund (DHT).

Introduction

Cervical Cancer

Cancer cell persistence can be attributed to any number of various factors. Infection by viruses, for example, causes changes in genotype that can disrupt cellular mechanisms triggering apoptosis. Many mechanisms exist to cause cancer, but some are more common than others. Cervical cancer remains the second most common cancer among females worldwide (20). The incidence rate varies by country, from 2 percent of women affected in the United States to as high as 50 percent of women affected in Guinea¹⁰. On average, 500,000 women develop cervical cancer annually, of which 99 percent arise from infection by the human papillomavirus, or HPV^{20, 18, 25}. Over 40 different strains of HPV exist that infect the genital areas of men and women¹⁸. Infected tissues include, but are not limited to, the cervix, vagina, penis, and rectum¹⁸. In particular, HPV strains 16 and 18 are especially high-risk because they cause approximately 70 percent of all cervical cancers^{18, 25}. Although 90 percent of HPV infections are generally resolved by attack from the body's immune system, HPV 16 and 18 are especially persistent and can result in cervical cancer¹⁸.

HeLa Cells as a Research Subject

Among the more widely-known types of HPV-infected cells associated with cervical cancer, HeLa cells (derived from the cervical tumor of Ms. Henrietta Lacks in 1951) have been extensively studied²⁷. The HeLa cell line, infected with HPV strain 18, is a particularly aggressive cancer cell and continues to thrive in laboratories worldwide today^{24, 27}.

The HeLa cells used in the current project consist of two readily apparent phenotypes: cells that express cytokeratin 8/18 (K8/18) intermediate filaments and cells that do not. Although these filaments are known to provide structural integrity to cells, in recent years it has been demonstrated that they also prevent apoptosis^{9, 28}.

Cytokeratin Intermediate Filaments

Cytokeratin 8/18 filaments are expressed in a multitude of epithelial organs. The proteins are essential structural components of epithelial cells that form a network of filaments within the cytoplasm¹². They help cells resist external pressures, maintain normal mitochondrial function, and regulate cellular functions such as mitosis, apoptosis, and cell signaling^{12, 14}. Because of their varied functions within the cell, cytokeratin filaments are suspected to play an integral role in cancer development^{7, 14, 17}.

Although the components of cytokeratins are highly conserved across species, they generally consist of at least 20 different gene products which can be arranged into a relatively acidic, Type I group (cytokeratins 9 through 20) and a neutral–basic, Type II group (cytokeratins 1 through 8)^{14, 17}. Cytokeratins exist as heterodimers in a 1:1 ratio of acidic and neutral-basic proteins, which then form filamentous polymers, such as cytokeratin 8/18^{14, 17}. Expression of these proteins is cell-specific and can be influenced by the degree of cell differentiation. For instance, as cancer cells become less differentiated, cytokeratin expression is often a useful diagnostic measure to identify carcinomas from other non-metastatic tumors⁷.

Effects of ERKs and Cytokeratins on Apoptosis

The ability of K8/18 filaments to interfere with immune-mediated apoptosis is considered one mechanism contributing to the development of epithelial cell carcinomas²³. The K8/18 filaments may prevent apoptosis through a variety of cellular mechanisms, including the upregulation of growth/differentiation pathways that counteract the effects of immune attack^{19, 6, 21}. Based upon previous work by others in which K8/18 filaments provided a protective effect in epithelial cells, we postulate that K8/18 filaments provide analogous protection from cytokine-mediated apoptosis in cervical cancer cells.

The extrinsic pathway of apoptosis in cells is predicated on the actions of cytokines such as Fas ligand to activate initiator and executor caspases within the cell, which in turn provoke cell death^{11, 32}. In Fas ligand-induced apoptosis, Fas ligand binds to its receptor to activate the Fas-associated death domain protein (FADD), that activates downstream proteins including caspase 8, and executor caspases 3 and 7^{11, 32}. Caspases 3 and 7 initiate apoptosis downstream along with Smac/DIABLO and other downstream pro-apoptotic proteins^{11, 15, 29}.

The mechanisms by which K8/18 filaments promote anti-apoptotic effects within cell are not entirely clear, but may include activation of the MAP kinase cascade to overcome Fas-induced death-signaling. The scaffolding provided by K8/18 filaments supports activation of Raf-1, the first kinase in the MAP kinase cascade²⁹. Raf-1 activates MEK 1/2, which then activates ERK 1/2 through phosphorylation²⁹. The collective actions of phosphorylated ERK lead to upregulated cell growth and differentiation downstream^{22, 6, 21}, which could counteract apoptotic events.

Previous Research

Decreased expression of phosphorylated extracellular-regulated kinases in HeLa cells renders them more sensitive to cytokine-induced apoptosis³⁰. Conversely, increased levels of phosphorylated ERK protect cells by suppressing caspase activation and subsequent apoptosis when exposed to these same cytokines³⁰.

To investigate this phenomenon further, an experiment was conducted in spring 2013 to determine if cytokeratin 8/18 filament expression in HeLa cells affects caspase activation after the cells are exposed to cytokines. Cultures of HeLa cells possessing and lacking cytokeratin 8/18 filaments (K+ and K- cells) were grown in 96-well plates for dose-response experiments involving several different cytokines. The cells were treated with TNF- α , TRAIL, or Fas agonist to determine the dose and type of cytokine most effective at inducing apoptosis. The cytokines TNF- α and TRAIL had essentially no effect on the two phenotypes, failing to induce caspase activity above non-cytokine-treated controls. However, Fas agonist did induce caspase activity in the cells, and K+ HeLa cells were more resistant to Fas-induced apoptosis than K- HeLa cells, as expected.

Immunodetection of ERK in K+ and K- HeLa cells

Following the above-described cytokine dose-response experiments, a subsequent experiment was conducted to determine the effect of K8/18 filaments on MAPK activation by measuring relative ERK expression. An initial set of samples collected in the summer 2013 indicated that ERK expression was present for both K+ and K- cells. Phosphorylated ERK expression was highest in K- cells treated with hEGF, but other treatment conditions did not appear to yield higher amounts of pERK for either phenotype. Unfortunately, subsequent

attempts to analyze ERK expression failed to provide consistent results. Explanations for the inconsistencies include inaccurate protein loading during electrophoresis, unsuitable antibody incubation solutions, and possibly other factors interfering with secondary antibody detection.

To address these problems, a standardized protocol was devised, and immunodetection experiments were repeated in fall 2013^{3,21}. In this run of western blots, the background was reduced and quantification of bands showed that K- cells exposed to hEGF exhibited greater ERK expression than any other treatment condition. We expected K+ cells to express greater amounts of ERK when treated with Fas agonist compared to K- cells treated with the same cytokine, but similar ERK expression was exhibited by both phenotypes after treatment with Fas agonist. Furthermore, pERK/totERK expression was inconsistent for K+ and K- negative control conditions, which indicates that conclusions cannot be drawn from these results alone due to the inconsistency of our control conditions.

In summary, there was no difference in ERK 1/2 expression between K+ and K- phenotypes, especially following treatment with Fas agonist. In fact the only indication that K8/18 filaments influenced MAPK activation in the HeLa cells in the current experiments stems from finding that hEGF-stimulated ERK 1/2 expression is greater in K- cells than K+ cells. This finding and inconsistencies in our control conditions necessitated that we repeat these experiments using alternate detection methods to determine if higher ERK 1/2 expression in K- cells following Fas agonist treatment was a result of inaccurate immunodetection or actual differences in ERK expression between the two phenotypes following Fas agonist treatment.

The above inconsistencies in methods and findings prompted us to repeat the Fas agonist and hEGF experiments using an alternative, in-cell western immunofluorescent assay for immunodetection^{1,2,4,21}. The rationale was that during western blot immunodetection, protein

loss occurs at many stages; including lysate transfer from culture plate to microfuge tube, removal of cell debris following centrifugation, and protein transfer from gel to PVDF membrane²¹. Any one of these losses could confound potential differences in protein detection between K+ and K- cell preps. The in-cell western assay, alternatively, offers more quantitative results with less protein loss²¹. Thus, additional experiments were conducted using this format for protein detection. Again, we hypothesized that K8/18 filaments promote anti-apoptotic effects within HeLa cells by activation of the MAPK cascade to overcome Fas-induced death-signaling. Measurement of phosphorylated extracellular-regulated kinases (pERK) was used as an indication of MAP kinase activation.

Expected Outcomes and Interpretations

The results of the in-cell western assays could partly determine if the western blotting experiments performed in summer 2013 were an anomaly or if they provide further evidence to reject the current hypothesis. In-cell western assays were anticipated to show elevated phosphorylated ERK 1/2 expression in K+ compared to K- HeLa cells, especially under conditions of Fas agonist treatment. An additional hope was that in-cell western assays would provide a more quantitative measure of ERK expression than traditional western blotting methods.

Materials and Methods

Western Blot Immunodetection

Two different HeLa strains, K+ and K- cells, were grown under sterile culture conditions. At 70 percent confluency, the cells were trypsinized and passaged into 6-well plates at a seeding concentration of 80,000 cells per mL until confluency of 70 percent or greater was again achieved. At this point the cultures were then treated with human epidermal growth factor (hEGF) as a positive control, Fas ligand as the experimental variable, or vehicle as the negative control (Appendix I). Following a 10 minute incubation period, the treatments were rinsed and removed from the cells. The cells were then scraped from the bottom of the wells in lysis buffer, lysed with a 28 gauge needle, and transferred into centrifuge tubes. After centrifugation the supernatant (containing proteins such as pERK) was separated from the cell debris in the pellet. Protein concentration of the supernatant was measured and the remaining sample was mixed with 5X or 2X Sample Buffer, Heated, and frozen at -80°C until further use (see detail-Appendix II).

Once samples were prepared as described above, they were loaded into polyacrylamide gels for protein separation via electrophoresis. Following size separation via electrophoresis, the proteins in the gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Appendix III). Following transfer, the membrane was incubated overnight at 4°C with primary antibodies that bind to phosphorylated ERK 1/2 and total ERK, and then probed with fluorescent secondary antibodies Dylight 680 and Dylight 800 for a half hour (Appendix IV). A LI-COR Odyssey infra-red imaging system was used to detect secondary antibodies at 700 nm and 800 nm

wavelengths. Protein content was quantified using Image Studio Lite software and MS Excel software aided in generation of statistical data and graphs.

In-Cell Western Immunodetection

In-cell western immunodetection was also performed using K+ and K- strains of HeLa cells. Three in-cell western assays were performed with treatments added in triplicate for each individual experiment. The HeLa cells were first grown as previously described prior to seeding in 96-well plates. At 70 percent confluency, the cells were treated with increasing doses of hEGF and without or with Fas agonist. The concentrations used were 5, 10, and 50 ng/mL EGF, 1 µg/mL Fas agonist, and 50 ng/mL hEGF plus 1 µg/mL Fas agonist (Appendix V). Within 10 minutes of treatment, the cells were rinsed and then fixed with 100% methanol in preparation for in-cell western assay (Appendix VI). Briefly, the fixative was removed and the wells were blotted dry. Subsequently the wells were rinsed three times for 5 minutes each with tris-buffered saline (TBS), blocked for one hour with blocking buffer and then incubated with ERK 1/2 and total ERK primary antibodies. The plate was incubated overnight at 4°C.

Following primary antibody incubation, the wells were rinsed three times for 5 minutes each with TBS and then fluorochrome-conjugated secondary antibody was added to the plate and incubated for one hour at room temperature in the dark (Appendix VI). The plate was again rinsed three times for 5 minutes each with TBS and then scanned with a LiCor Odyssey infra-red imaging system at 700 and 800 nm wavelengths to visualize phosphorylated ERK and total ERK proteins. Proteins were quantified using Image Studio Lite software and MS Excel software helped generate statistical data and graphs.

Results

The western blot immunodetection showed markedly increased pERK expression in K⁻ HeLa cells compared to K⁺ HeLa cells, especially under control and EGF-stimulated conditions (Figures 1 and 2). For both K⁺ and K⁻ cells, Fas agonist-induced pERK expression was comparable (Figure 2).

Subsequent experiments using in-cell western assays revealed similar trends to the above. That is, K⁻ HeLa cells exhibited higher pERK expression than K⁺ HeLa cells, but only in response to hEGF stimulation (Figures 3 and 4). Expression of pERK was again similar between K⁺ and K⁻ HeLa cells following Fas agonist treatment but was less than that observed for control or hEGF treatment conditions (Figure 4). The combination treatment of Fas agonist and hEGF restored pERK expression in both cell types to pretreatment levels (Figure 4). Additionally, the overall relative expression of pERK by in-cell western assay was less than that observed by traditional western immunoblot analysis (Figures 1 and 3).

Discussion

Cytokeratin 8/18 filaments protect cells against cytokine-induced death, but their role in HeLa cervical cancer cells remains unclear. The current study confirmed that Fas agonist is more potent in killing K- HeLa cell than other cytokines, including TNF and TRAIL²⁸. Moreover, although the activation of the MAP kinase pathway is implicated, it does not appear to play a direct role in cytokeratin-mediated protection as reported previously in another cell model^{13, 19, 26}. Similar expression of phosphorylated ERK was observed in both K+ and K- HeLa cells following exposure to Fas agonist, indicating little to no effect of cytokeratin filaments on the MAP kinase pathway. Alternatively, other signaling pathways, such as the PI3K/Akt pathway, may be the primary anti-apoptotic pathway activated by cytokeratin filament in HeLa and other types of epithelial cells following Fas agonist treatment⁵. Future experiments that focus on PI3K/Akt pathway may reveal that K8/18 expression counteracts Fas-mediated apoptosis through mechanisms anticipated of the MAP kinase pathway.

Regardless of the above observations, clear evidence that differences in pERK expression exist between K+ and K- HeLa cells was presented. More specifically, using hEGF as a positive control treatment for phosphorylated ERK expression, we determined that 5 ng/mL of hEGF elicits much greater expression of pERK in K- than K+ cells^{8, 31}. The results suggest that cytokeratin filaments may actually impair the MAP kinase pathway in some manner that is not decipherable from the current experiments. Repeating these experiments and directly determining the relationship between the cytokeratin filaments and MAP kinase is one possible direction for future research. Using a cytokine other than Fas agonist or a growth factor related to

but not hEGF might also help discern whether the K8/18 filaments inhibit some novel aspect of the MAP kinase pathway.

Fas agonist treatment, known to induce cell death in K- HeLa cells, combined with hEGF negated the effects of Fas agonist alone on pERK expression. This indicates that the effects of Fas activation are possibly counteracted by cyokeratin filaments and additional pathways beyond MAPK than previously hypothesized. Sarcomas, for example, have been shown to avoid Fas ligand-induced apoptosis by stimulating secretion of EGF¹⁶. While it is currently unclear whether HeLa cells utilize hEGF to avoid Fas-induced apoptosis, perhaps future experiments could focus on this phenomenon.

Overall, the results of the current experiments did not support the hypothesis that K8/18 filaments protect HeLa cells from Fas-mediated apoptosis through activation of the MAP kinase pathway. Instead, we suggest these cells are protected by another mechanism, possibly activation of the PI3K/Akt pathway, or perhaps some yet undiscovered mechanism that ensures the metastatic potential of this cervical cancer cell.

Figures

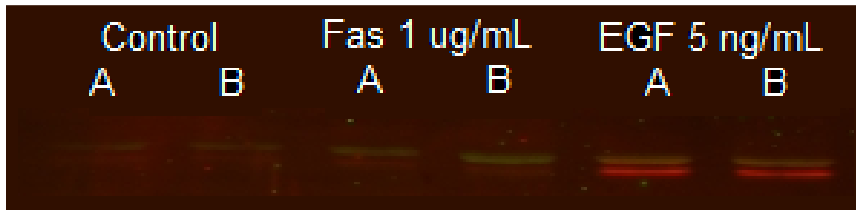


Figure 1: Representative immunoblot for the detection of phosphorylated ERK 1/2 (pERK; red bands) and total ERK (totERK; green bands) in K- HeLa cell lysates. Note higher expression of pERK in EGF-treated samples. Quantification of pERK expression relative to totERK in K+ vs. K- cells for the three experiments is depicted below.

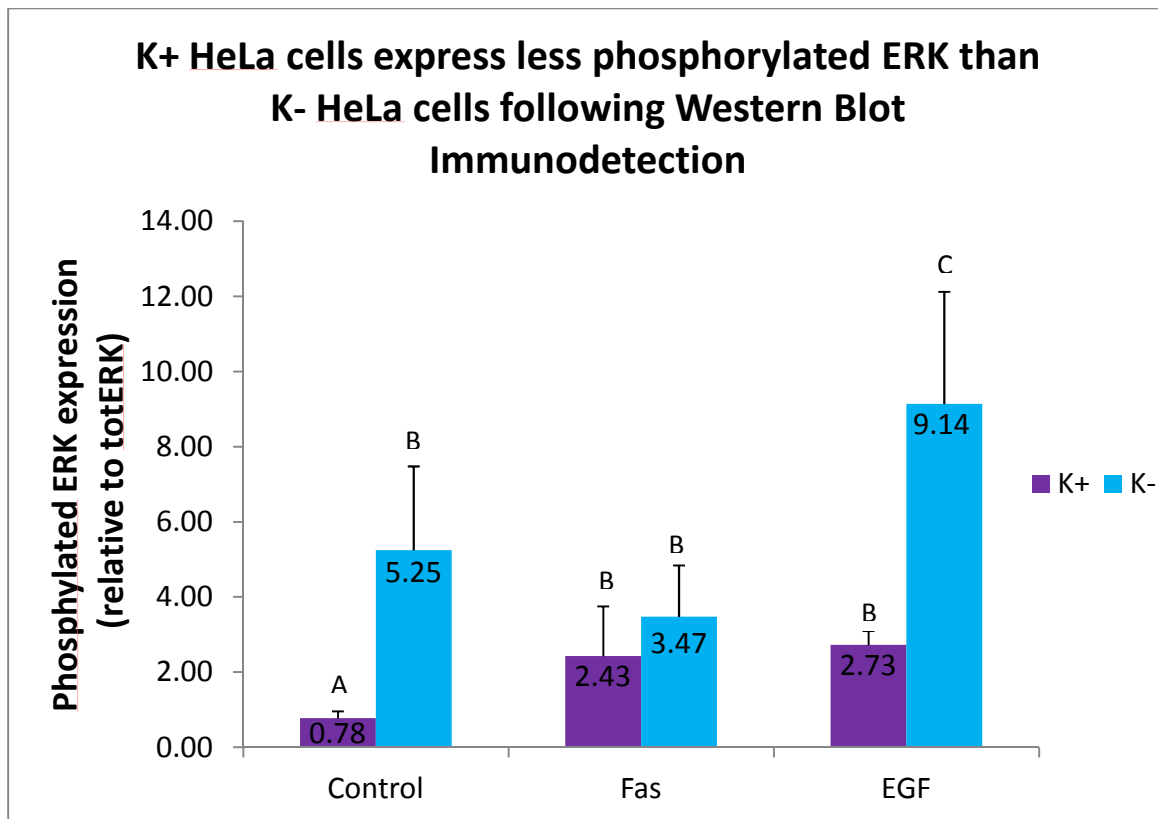


Figure 2. Effects of Fas agonist (1ug/ml) and EGF (5ng/ml) on phosphorylated ERK (pERK) and total ERK (totERK) expression in K+ and K- HeLa cells. K+ cells had less pERK expression compared to K- cells, especially under control and EGF-stimulated conditions. Treatments in each experiment were performed in duplicate, and the experiment was repeated three times. Different letters denote differences among treatment groups ($p < 0.05$).

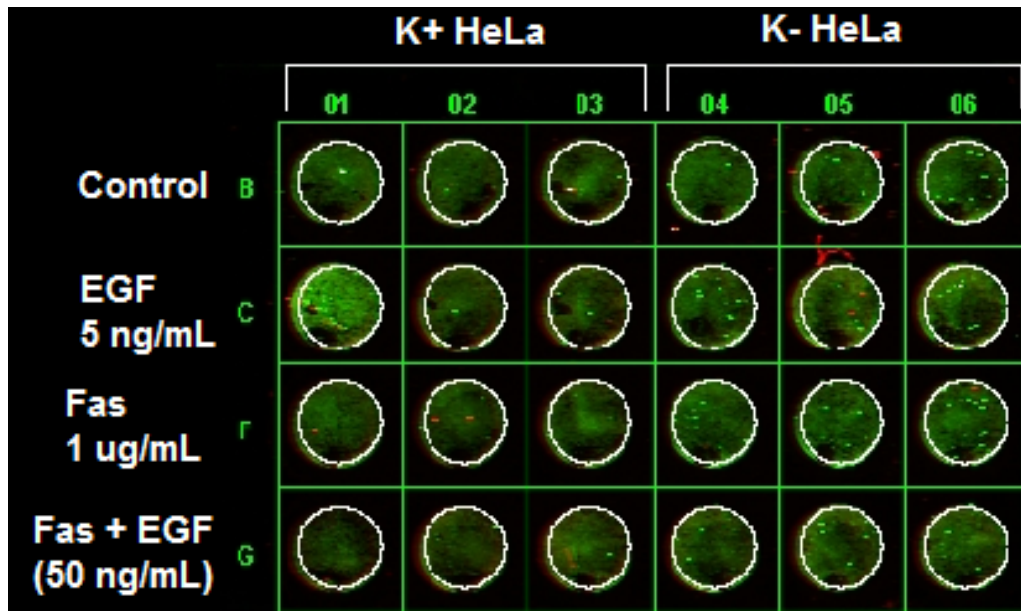


Figure 3: Representative In-Cell Western assay for phosphorylated ERK 1/2 and total ERK expression in K+ and K- HeLa cells. Phosphorylated ERK 1/2 proteins detected as red; total ERK expression detected as green. Quantification of pERK expression relative to totERK in K+ vs. K- cells for the three experiments is depicted below.

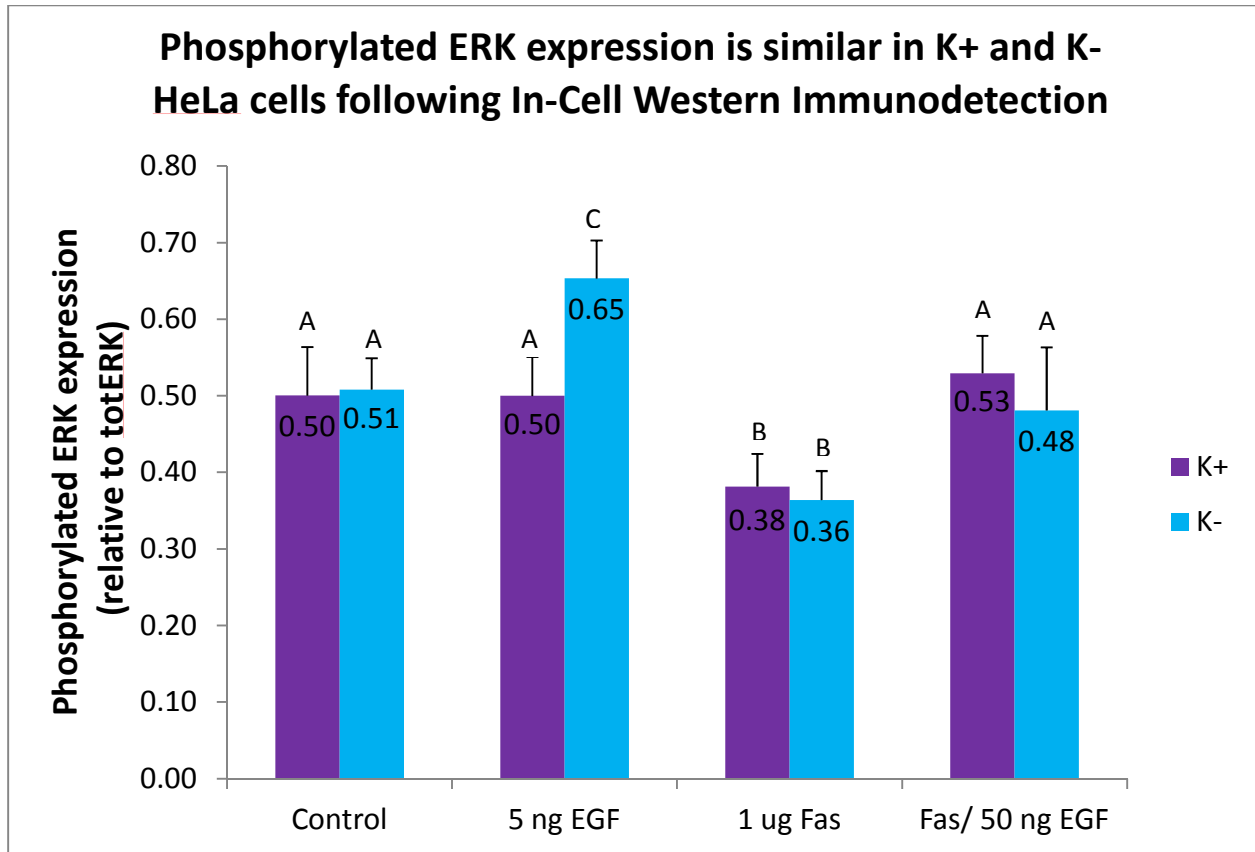


Figure 4. Effects of Fas agonist (1ug/ml) and EGF (50ng/ml) on phosphorylated ERK (pERK) and total ERK (totERK) expression in K+ and K- HeLa cells. Relative in-cell expression of pERK was similar in K+ and K- cells, was impaired by Fas agonist, but rebounded in response to EGF stimulation (50ng/ml). Treatments were performed in duplicate, and the experiment was repeated three times. Different letters denote differences among treatment groups ($p < 0.05$).

References

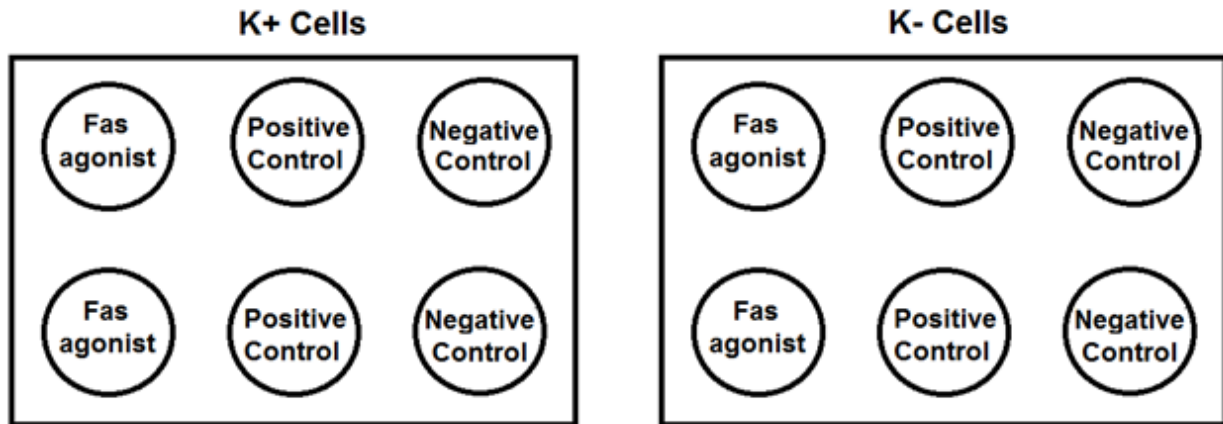
1. In-Cell Western [Internet] <http://www.cellsignal.com/ddt/licor.html>. Internet: Cell Signaling Technology; c2013 [cited 2013 November 17]. Available from: <http://www.cellsignal.com/common/content/content.jsp?id=in-cell-western>.
2. Application Overview: In-Cell Western Assay [Internet] <http://gcf.pbrc.edu/docs/Odyssey/In-Cell%20Western%20Overview.pdf>. Internet: LI-COR Biosciences; c2013 [cited 2013 November 17]. Available from: <http://gcf.pbrc.edu/docs/Odyssey/In-Cell%20Western%20Overview.pdf>.
3. Technical Note: Western Blot and In-Cell Western Assay Detection Using IRDye Subclass Specific Antibodies [Internet] http://biosupport.licor.com/docs/TechNote_SubclassSpecAb.pdf. Internet: LI-COR Biosciences; c2011 [cited 2013 November 17]. Available from: http://biosupport.licor.com/docs/TechNote_SubclassSpecAb.pdf.
4. In-Cell Western Protocol [Internet] http://www.cellsignal.com/support/protocols/In_Cell_Western.html. Internet: Cell Signaling Technology; c2010 [cited 2013 November 17]. Available from: <http://www.cellsignal.com/contents/resources-protocols/in-cell-western-protocol/in-cell-western-protocol>.
5. Abreu MT, Arnold ET, Chow JYC, and Barrett KE. Phosphatidylinositol 3-kinase-dependent pathways oppose fas-induced apoptosis and limit chloride secretion in human intestinal epithelial cells. *The Journal of Biological Chemistry* 2001;276(50):47029-37.
6. Aoki K, Yamada M, Kunida K, Yasuda S, Matsuda M. Processive phosphorylation of ERK MAP kinase in mammalian cells. *PNAS* 2011;108(31):12675-80.
7. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathology* 2002(40):403–39.
8. Dschietzig T, Bartsch C, Richter C, Laule M, Baumann G, Stangl K. Relaxin, a pregnancy hormone, is a functional endothelin-1 antagonist. *Circulation Research* 2003;92:32-40.
9. Eriksson JE, Dechat T, Grin B, Helfand B, Mendez M, Pallari H, Goldman RD. Introducing intermediate filaments: From discovery to disease. *Journal of Clinical Investigation* 2009 07;119(7):1763-71.
10. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available from: <http://globocan.iarc.fr>, accessed on 2/2/2013.

11. Ferreira KS, Kreutz C, Macnelly S, Neubert K, Haber A, Bogyo M, Timmer J, Borner C. Caspase-3 feeds back on caspase-8, bid and XIAP in type I fas signaling in primary mouse hepatocytes. *Apoptosis* 2012 05;17(5):503-15.
12. Fuchs E, Weber K. Intermediate filaments: structure, dynamics, function, and disease. *Annu Rev Biochem* 1994(63):345–82.
13. Gilbert S, Ruel A, Loranger A, Marceau N. Switch in fas-activated death signaling pathway as result of keratin 8/18-intermediate filament loss. *Apoptosis* 2008 12;13(12):1479-93.
14. Hesse M, Magin TM, Weber K. Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudogenes related to keratin genes 8 and 18. *J Cell Sci* 2001(114):2569–75.
15. Hui KKW, Kanungo AK, Elia AJ, Henderson JT. Caspase-3 deficiency reveals a physiologic role for Smac/DIABLO in regulating programmed cell death. *Cell Death and Differentiation* 2011 11;18(11):1780-90.
16. Joyner DE, Jones KB, Lessnick SL, Schiffman JD, Randall RL. (2011) Potential for modulation of the fas apoptotic pathway by epidermal growth factor in sarcomas. *Sarcoma* 2011:847409.
17. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 1982(31):11–24.
18. Lee SL. A mathematical model of human papillomavirus (HPV) in the united states and its impact on cervical cancer. *Journal of Cancer* 2012(1):262.
19. Leifeld L, Kothe S, Söhl G, Hesse M, Sauerbruch T, Magin TM, Spengler U. Keratin 18 provides resistance to fas-mediated liver failure in mice. *European Journal of Clinical Investigation* 2009 06:39(6):481-8.
20. Liao S, Lee W, Chen H, Chuang L, Pan M, Chen C. Baseline human papillomavirus infection, high vaginal parity, and their interaction on cervical cancer risks after a follow-up of more than 10 years. *Cancer Causes & Control* 2012(5):703.
21. Magi B, Liberatori S. Immunoblotting techniques. *Methods in Molecular Biology* 2005 (295):227-54.
22. Myoung WL, Jae HB, Hyun JL, Do YL, Wan SJ, Yong SK, Soon CP, Kyung YK, Won BL, Kim SS. The activation of ERK1/2 via a tyrosine kinase pathway attenuates trail-induced apoptosis in HeLa cells. *Cancer Investigation* 2005 11;23(7):586-92.
23. Oshima RG. Apoptosis and keratin intermediate filaments. *Cell Death & Differentiation* 2002 05;9(5):486.

24. Picken RN, Yang HL. The integration of HPV-18 into HeLa cells has involved duplication of part of the viral genome as well as human DNA flanking sequences. *Nucleic Acids Research* 1987 12/10;15(23):10068.
25. Schiffman, Wentzensen, Sholom Wacholder, Kinney, Gage, Castle J. Human papillomavirus testing in the prevention of cervical cancer. *Natl Cancer Inst.* 2011 March 2; 103(5): 368–383.
26. Schoeberl B, Eichler-Jonsson C, Gilles ED, Muller G. Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors. *Nature Publishing Group* 2002;20(4):370-5.
27. Skloot R. *The Immortal Life of Henrietta Lacks*. New York (NY): Random House, Inc.; 2011.
28. Sullivan BT, Cherry, JA, Sakamoto H, Henkes LE, **Townson DH**, Rueda, BR. Cytokeratin-18 (CK18) expression inhibits cytokine-induced death of cervical cancer cells. *International Journal of Gynecologic Cancer* 2010 20:1474-1481.
29. Tian T, Song J, Khanin R. Mathematical modelling of the MAP kinase pathway using proteomic datasets. *Plos One* 2012 08;7(8):1-12.
30. Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE. MAPK/ERK overrides the apoptotic signaling from fas, TNF, and TRAIL receptors. *Journal of Biological Chemistry* 2001 05/11;276(19):16484-90.
31. van der Westhuizen ET, Werry TD, Sexton PM, Summers RJ. The relaxin family peptide receptor 3 activates extracellular signal-regulated kinase 1/2 through a protein kinase C-dependent mechanism. *Molecular Pharmacology* 2007;71(6):1618-29.
32. Wang DH, Hu JR, Wang LY, Hu YJ, Tan FQ, Zhou H, Shao JZ, Yang WX. The apoptotic function analysis of p53, Apaf1, Caspase3 and Caspase7 during the spermatogenesis of the chinese fire-bellied newt *cynops orientalis*. *Plos One* 2012 06;7(6):1-20.

Appendix I

Treatment Diagram for 6-well Culture Plates



The positive control denotes EGF (epidermal growth factor) in 5 ng/mL concentration. Fas agonist treatment is 1 ug/mL.

Appendix II

HeLa Lysate Protocol (Preparing K⁺ and K⁻ Lysates for Fluorescent Western Detection of pERK and total ERK)

Materials:

rhEGF

Fas ligand

HeLa cells grown to 70% (or greater) confluence in 6-well plate

Ice-cold PBS

Ice-cold lysis buffer-- 10 mM Tris-HCl; 1 mM EGTA; 100 mM NaCl; 1% Triton X; 0.5% Nonidet P-40, pH 7.4

Directions:

- 1.) Aspirate conditioned medium from 6-well plate and replace with fresh medium.
- 2.) Add FasL (1 ug/mL concentration) and rhEGF (5 ng/mL concentration) in 10 uL spike to appropriate wells.
- 3.) Incubate plate at 37°C and 5% CO₂ for 10 minutes.
- 4.) At the end of 10 minutes, rinse cells with ice-cold PBS (non-sterile).
- 5.) Add 500ul/well ice-cold lysis buffer containing protease inhibitor (10 uL/mL) and phosphatase inhibitor (10 uL/mL) cocktails to wells.
- 6.) Scrape cells from wells, concentrating lysate into small volume and aspirate into microfuge tubes
- 7.) Further lyse cells by passaging through a 28-gauge needle.
- 8.) Centrifuge lysates briefly (pulse spin microfuge) to ensure a pellet forms.
- 9.) Sonicate (vortex) samples for 15 seconds each.
- 10.) Perform BCA assay to determine protein concentration.
- 11.) Centrifuge samples for 5 minutes at 13,000 x g to pellet cell debris.
- 12.) Denature proteins by heating samples at 100°C for 5 minutes.
- 13.) For low protein concentration, add 1 part 5X sample buffer to 4 parts sample. For high protein concentration, add equal amount of 1X sample buffer to sample.
- 14.) Store in freezer at -80°C until use.

SDS-PAGE:

Total protein (25-30 uL) was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred (75 V for 2 hours) to polyvinylidene fluoride (PVDF) membranes (Immobilon-FL, Millipore, Billerica, MA). See Western Blotting Protocol for further details.

Appendix III

Western Blotting Protocol

(Adopted from Yao Lab's "Odyssey Western Blotting")
Amanda Berger

Electrophoresis:

1. Prepare acrylamide gel according to directions located next to chemical hood (if those are not available, follow Gel Casting Protocol from 5/14/13).
2. Remove comb carefully from gel to ensure that wells don't break.
3. Lubricate the grey insulators on the electrophoresis chamber to ensure a good seal.
4. Remove gel from gel caster and clip onto electrophoresis chamber.
5. Pour tank buffer into the middle of the chamber to test if the apparatus is leaking. Once it has been determined that the seal is good, pour buffer into the bottom of the chamber; do not put buffer in between empty plate and chamber.
6. Load samples (20 uL/well) onto gel carefully; do not use the two outer wells on both sides of gel.
7. Attach the cover and plug in the electrodes. Turn on the power source and adjust settings for use. Run the gel at max voltage (all the way clockwise) and current at 20 for one hour.

Gel Transfer:

1. Cut the PVDF membrane and blotting papers (6) to the size of the gel.
2. Incubate the PVDF membrane in 100% methanol for 5 minutes.
3. Next, incubate the PVDF membrane in Towbin transfer buffer for 5 minutes.
4. When the gel is finished running, remove the gel carefully from the cassette and measure it. Cut off one corner of the gel and place the gel in Towbin transfer buffer. Shake the gel in buffer for 5 minutes.

Preparation of the Gel Sandwich and Protein Transfer:

1. Tape a mylar mask in place on the mesh screen in the transfer unit.
2. Dip a piece of blotting paper in the transfer buffer until it is just wet; place this paper over the mylar mask. Ensure that the blotting paper is larger than the hole in the mylar mask.
3. Use a test tube to remove any air bubbles between the paper and transfer unit.
4. Repeat steps 2 and 3 with two more pieces of blotting paper.
5. Place the membrane on the blotting paper and remove any air bubbles.
6. Carefully place the gel on the membrane; try not to readjust its position as proteins may transfer as soon as the gel is placed. Remove any air bubbles.
7. Dip and place 3 more blotting papers on the sandwich. Remove air bubbles as the blotting papers are added. The sandwich should be as follows: 3 blotting papers; 1 membrane; 1 gel; 3 blotting papers.
8. Put the cover on the apparatus and plug in the electrodes to the power source.
9. Set the voltage all the way clockwise (max) and the current to 36 and run for 45 minutes.

Appendix IV

Immunodetection Protocol

(After protein transfer to membrane)

Amanda Berger

Materials:

Blocking Buffer: For 50 mL, add 2.5 g nonfat dry milk to 50 mL TBS

TBST: For 1 L, add 1 mL Tween 20 to 1000 mL TBS

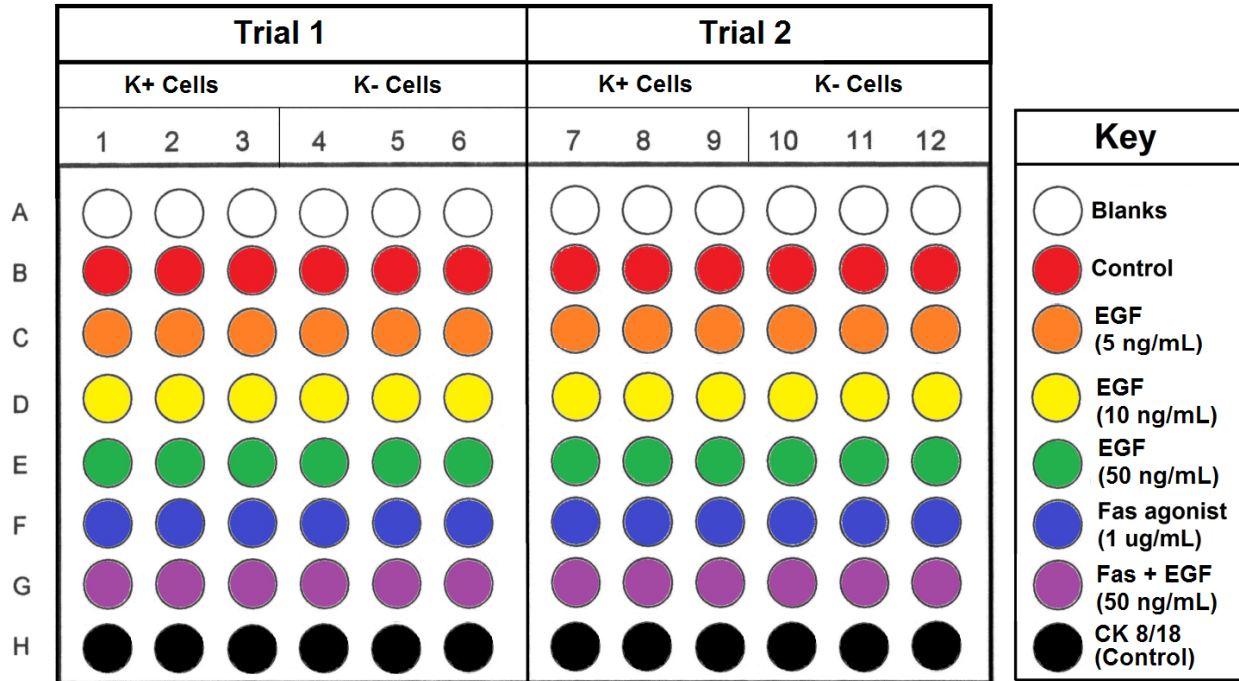
Primary Antibody Dilution Buffer: 10 mL TBST, 0.5 g BSA, 5 uL phospho-p44/42 MAPK (ERK 1/2) rabbit mAb, and 5 uL p44/42 MAP Kinase (Total ERK) mouse mAb (1:2,000 dilution)

Secondary Antibody Dilution Buffer: 20 mL TBST, 1 g nonfat dry milk, 1 uL Dylight 800 (anti-Total ERK, 1:20,000 dilution), and 1.5 uL Dylight 680 (anti-ERK 1/2, 1:15,000 dilution)

1. Following protein transfer (from Western Blotting Protocol), rinse the PVDF membrane in 100% methanol for 1 minute.
2. Discard methanol and rinse the membrane with water.
3. Discard water and rinse the membrane in 1xTBS for 2 minutes and leave the membrane in TBS until further steps (never let the membrane DRY!)
4. Place the membrane in 30 mL Blocking Buffer (TBS with nonfat dry milk) for 1 hour at room temperature with gentle shaking.
5. Dilute the primary antibodies as suggested on the product sheet into 10 mL of primary antibody buffer with Tween-20. Add this buffer to the membrane and incubate at 4°C overnight with gentle shaking.
6. Pour off primary antibody dilution buffer (recover buffer; do not discard).
7. Wash the membrane in 1xTBST 3 times for 5 minutes each with gentle shaking at room temperature.
8. Pour off 1xTBST and add 20 mL secondary antibody diluted as suggested on the product sheet.
9. Incubate the membrane in the Western Blot cylinder for ½ hour in the dark at room temperature. (Make sure to protect the membrane from light from now on.)
10. Wash the membrane with 1xTBST 3 times for 5 minutes each in the dark at room temperature.
11. The membrane should be kept wet until read on the LiCor Odyssey machine.
12. The membrane can now be dried and stored in between blotting papers in tin foil in the fridge.

Appendix V

Treatment Diagram for 96-well Culture Plates



One 96-well plate yields two trials under ideal conditions. CK 8/18 control wells were not treated with cytokine nor growth factor.

Appendix VI

In-Cell Western Protocol (adapted 1/14/14)

Amanda Berger

Culture and Treatment:

K+ and K- HeLa cells were cultured until 70-80% confluent. Cells were treated with 5 ng/mL EGF, 10 ng/mL EGF, 50 ng/mL EGF, 1 ug/mL Fas agonist (CH11), and 50 ng/mL EGF + 1 ug/mL Fas for non-control conditions. Following treatment, cells were incubated for 10 minutes in incubator (95% humidity, 37°C, 5% CO₂). Immediately after treatment, cells were fixed and permeabilized.

Fixation and permeabilization:

Cells were fixed and permeabilized with 100% methanol (50 uL/well). Growth medium was removed from the microplate by inversion on a paper towel then immediately fixed and permeabilized for 10 minutes at room temperature with gentle shaking with 50 uL ice-cold methanol per well (methanol added down the sides of the wells to avoid cell detachment). Methanol was removed from the plate by inversion on a paper towel then rinsed three times for 5 minutes with gentle shaking with 1X PBS (100 uL/well).

In-Cell Western

Cells were blocked for one hour at room temperature with 50 uL blocking buffer (1X PBS / 5% normal goat serum / 0.3% Triton X-100) per well. After blocking buffer, cells were washed 3 times (5 minutes) with 1X PBS. Cells were probed overnight at 4C with 50 uL/well primary antibody cocktail against Phospho-p44/42 MAPK (ERK 1/2, Cell Signaling Technology, Danvers, MA) diluted 1:200 and p44/42 MAP Kinase (L34F12, Cell Signaling Technology, Danvers, MA) in antibody dilution buffer (1X PBS / 1% BSA / 0.3% Triton X-100). Control wells were probed with K18 (CY90, Sigma Aldrich, St. Louis, MO) diluted 1:800 and β -Actin (13E5, Cell Signaling Technology, Danvers, MA) diluted 1:200 in antibody dilution buffer (1X PBS / 1% BSA / 0.3% Triton X-100) to ensure appropriate K 8/18 expression in the two phenotypes. Following three washes for 5 minutes with PBS, cells were incubated for 1 hour at room temperature in the dark with a cocktail of fluoro-chrome-conjugated anti-mouse and anti-rabbit secondary antibodies (anti-mouse DyLight 800 and anti-rabbit DyLight 680, Cell Signaling Technology, Danvers, MA) diluted 1:2000 and 1:1000, respectively, in antibody dilution buffer. Following three washes with PBS, the microplate was scanned using the LI-COR® Odyssey® Classic Infrared Imaging Scanner to detect and quantify K18 expression relative to β -Actin (LI-COR®, Lincoln, NE).



Effects of keratin filaments on ERK signaling during Fas-induced death of cervical cancer (HeLa) cells

Amanda Berger and David H. Townson PhD

Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire



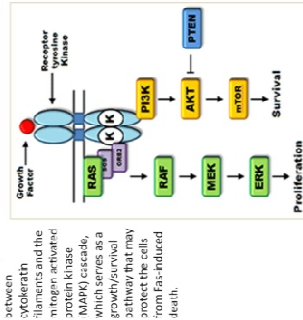
Abstract

Survival of cancer cells is influenced by a variety of factors including physical elements such as keratin filaments. We know HeLa cells containing or lacking keratin 8/18 intermediate filaments (K+ and K- cells, respectively) are more sensitive to the death-inducing effects of Fas agonist compared to other cyclin-like such as tumor necrosis factor alpha (TNF- α) or TNF-related apoptosis-inducing ligand. Additionally, K- cells are more sensitive to Fas-induced death than K+ cells. In the current study we tested the hypothesis that keratin filaments associate with the mitogen-activated protein kinase (MAPK) cascade to protect cells from Fas-induced death. To do this, K+ and K- cells were exposed to Fas agonist and then, to human epidermal growth factor (EGF), known to stimulate MAPK. Fas agonist downregulated MAPK phosphorylation (pERK) in K+ cells, but not in K- cells. pERK phosphorylation (pERK) was also downregulated in K+ cells compared to non-EGF-treated controls, as 3 exerts a protective effect on K+ cells. Conversely, EGF (500 ng/ml) reversed this outcome, but again did so equally in K+ and K- cells. Intriguingly, K+ cells were more responsive to EGF stimulation alone than K- cells, regardless of EGF dose (pERK ~27% higher in K+ than K- cells, n = 3 repeats). Thus, certain 8/18 filaments do associate with MAPK to influence ERK phosphorylation and cell survival. Supported by the Hamel Center for Undergraduate Research (AB) and the COLSA Karatekas Fund (DHT).

Introduction

Cervical cancer is the second-leading cause of cancer-related death among women worldwide, with over 39k in 2018. It is the 11th leading cause of cancer death in the US. One of the hallmarks of cervical cancer cells is the ability to evade immune attack, thereby enabling HPV to replicate. Interestingly, the immortal cervical cancer cell line, HeLa, consists of at least two different types of cells in terms of phenotype: some cells express cytokeratin 8/18 intermediate filaments (K+ cells), while others lack these filaments (K- cells). While it is known that cytokeratin expression is clinically diagnostic of cervical cancer and tumor progression, the aims of the current study were to determine the relationship between

MAP Kinase and Akt Pathways⁴



cytokeratin filaments and the mitogen-activated protein kinase (MAPK) pathway, which serves as a growth/survival pathway that may protect the cells from Fas-induced death.

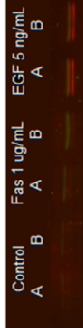
Contact: Amanda Berger (aj154@unh.edu) and David H. Townson (dave.townson@unh.edu)

Immunoblot Experiments

Methods and Materials

- HeLa cells (K+ and K- phenotype) grown to 70% confluency
- Cells exposed to Fas agonist (1 μ g/ml) or human epidermal growth factor (hEGF; 5 ng/ml)
- Cells lysed and proteins separated by SDS-PAGE
- Proteins transferred to PVDF membrane and exposed to antibodies for the detection of phosphorylated ERK1/2 and total ERK
- ERK1/2 and total ERK visualized by infrared detection using the LCor Odyssey system
- ERK1/2 and total ERK bands quantified using Image Studio Lite software.
- Experiments independently replicated three times (n = 3 experiments).

Results



Representative immunoblot for the detection of phosphorylated ERK1/2 (pERK; red bands) and total ERK (tERK; green bands) in K+ HeLa cells. Note higher expression of pERK in EGF-treated samples. Quantification of pERK expression relative to tERK in K+ vs. K- cells for the three experiments is depicted below.

K+ HeLa cells express less phosphorylated ERK than K- HeLa cells

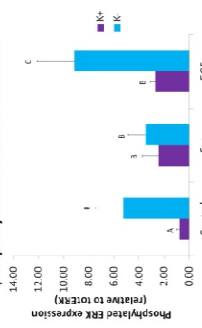


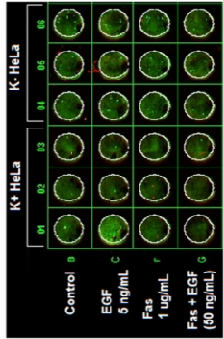
Figure 2. Effects of Fas agonist (1 μ g/ml) and EGF (5 ng/ml) on phosphorylated ERK (pERK) and total ERK (tERK) expression in K+ and K- HeLa cells. K+ cells had less pERK expression compared to K- cells, especially under control and EGF-stimulated conditions. Treatments in each experiment were performed in duplicate, and the experiment was repeated three times. Different letters denote differences among treatment groups (p < 0.05).

In-Cell Western Experiments

Methods and Materials

- K+ and K- HeLa cells seeded into 96-well plates
- Cells were exposed to Fas agonist (1 μ g/ml), and/or hEGF at increasing doses (5, 10, and 50 ng/ml)
- Cells fixed, permeabilized, and exposed to antibodies for phosphorylated ERK1/2 and total ERK
- Proteins were visualized by infrared detector on the LCor Odyssey system
- Experiments independently replicated three times (n = 3 experiments).

Results



Representative in-cell Western assay for phosphorylated ERK1/2 and total ERK expression in K+ and K- HeLa cells. Phosphorylated ERK1/2 proteins detected as red; total ERK expression relative to tERK in K+ vs. K- cells for the three experiments is depicted below.

Phosphorylated ERK expression is similar in K+ and K- HeLa cells

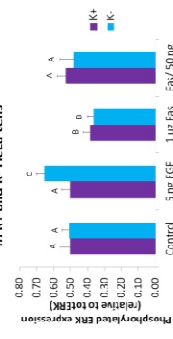


Figure 4. Effects of Fas agonist (1 μ g/ml) and EGF (50 ng/ml) on phosphorylated ERK (pERK) and total ERK (tERK) expression in K+ and K- HeLa cells. Relative in-cell Western expression of pERK was similar in K+ and K- cells, was increased by Fas agonist, but rebounded in response to EGF stimulation (50 ng/ml). Treatments were performed in duplicate, and the experiment was repeated three times. Different letters denote differences among treatment groups (p < 0.05).

Results (cont'd)

- Immunoblots indicated less pERK expression in K+ HeLa cells compared to K- HeLa cells
- In-Cell Western assays indicated similar pERK expression in K+ and K- HeLa cells
- EGF-stimulated pERK expression was more evident in cell lysates compared to in-cell detection, especially in K- HeLa cells
- Differences in pERK detection using immunoblotting versus in-Cell Western assay (pERK vs. tERK cells)
- Treatment with Fas agonist, known to induce cell death in K- HeLa cells, negated the EGF-stimulated increase in pERK expression

Summary

- K+ HeLa cells were less responsive to EGF stimulation than K- HeLa cells in terms of pERK expression (Figures 2 and 4)
- A high dose of EGF (50 ng/ml) reversed the negative effect of Fas agonist on pERK expression equally in K+ and K- HeLa cells (Figure 4)
- The above observations, along with noted differences in pERK expression attributed to detection methods (i.e., immunoblots vs. In-Cell Western), indicate that filaments are associated with MAPK to potentially influence cell survival

Conclusions

The observation that K+ HeLa cells express less pERK than K- HeLa cells (Figure 2) was unexpected, and suggests that keratin filaments do not promote cell survival via expression of ERK1/2 proteins as previously hypothesized. However, in the current study detection of pERK was inconsistent, and was in part influenced by HeLa cell phenotype (K+ vs. K-), but also by methodological approach (cell lysates for immunoblotting vs. fixed, intact cells for In-Cell Western). Future experiments, possibly utilizing other strains of cervical cancer cells, are needed to resolve these issues, and to help determine the nature of the relationship between keratin filament expression and the phosphorylation of ERK1/2 in the MAPK pathway.

References and Acknowledgements

- Lee S. A mechanistic model of human papillomavirus (HPV) in the cellular state and its impact on cervical cancer. *Journal of Cancer* 2021;12:262.
- Jiao Y, Lee W, Chen H, Zhang L, Pan M, Chen C. Baseline human papillomavirus (HPV) infection and its association with cervical cancer. *Journal of Cancer* 2019;10:108.
- Schmitt W, Wertzmann, Stefan W, Hildner, Krenn, Giger, C, Geller, J, Human papillomavirus infection and its role in cervical cancer. *Frontiers in Oncology* 2019;9:108.
- Wu Z, Zhang and George A, Vande Woude (2013). Role of ERK2 in the regulation of receptor tyrosine kinase signaling: sharing Akt via integrin-mediated cross-talk. *Trends in Cell Science* 2013;13:154-163.

Supported by the Hamel Center for Undergraduate Research (AM) and the COLSA Karatekas Fund (DHT).